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Rare Nav1.7 variants associated with painful diabetic peripheral neuropathy

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Abstract

Diabetic peripheral neuropathy (DPN) is a common disabling complication of diabetes. Almost half of DPN patients develop neuropathic pain for which current analgesic treatments are inadequate. Understanding the role of genetic variability in the development of painful DPN is needed for improved understanding of pain pathogenesis, for better patient stratification in clinical trials and to target therapy more appropriately. Here we examined the relationship between variants in the voltage gated sodium channel Nav1.7 and neuropathic pain in a deeply phenotyped cohort of patients with DPN. While no rare variants were found in 78 participants with painless DPN, we identified twelve rare Nav1.7 variants in ten (out of 111) study participants with painful DPN. Five of these variants had previously been described in the context of other neuropathic pain disorders and seven have not previously been linked to neuropathic pain. Those patients with rare variants reported more severe pain and greater sensitivity to pressure stimuli on quantitative sensory testing. Electrophysiological characterization of two of the novel variants (M1852T and T1596I) demonstrated gain of function changes as a consequence of markedly impaired channel fast inactivation. By using a structural model of Nav1.7 we were also able provide further insight into the structural mechanisms underlying fast activation and the role of the C-terminal domain in this process. Our observations suggest that rare Nav1.7 variants contribute to the development neuropathic pain in patients with diabetic peripheral neuropathy. Their identification should aid understanding of sensory phenotype, patient stratification and help target treatments effectively.

Keywords: diabetic peripheral neuropathy, neuropathic pain, voltage gated sodium channel Nav1.7, genetics, electrophysiology

Introduction

Diabetes mellitus is a common chronic disease that affects 415 million people worldwide (IDF Diabetic Atlas) and the numbers are expected to rise arriving to 642 million by 2040. Diabetic peripheral neuropathy (DPN) is one of the most frequent long-term complications of diabetes affecting 30-50% of patients and is associated with significant morbidity [26; 59]. Up to 50 % of the patients suffering from DPN will develop neuropathic pain which is typically expressed as spontaneous pain but, in a minority, also includes evoked pain such as brush evoked allodynia [2; 9; 62] . Painful DPN can have a major deleterious impact on the patients` quality of life and, unfortunately, the current first line analgesics show modest efficacy and poor tolerability [28]. In order to develop new and better target existing therapies for painful DPN we need to improve our understanding of neuropathic pain pathophysiology and its individual variability in DPN.

Risk factors for the development of painful DPN include older age, poor glycaemic control and more severe neuropathy. However, genetic factors have been relatively under-explored [32]. Here investigate the relationship between variants in the voltage gated sodium channel Nav1.7 and painful DPN.

The rationale for studying Nav1.7 is that this voltage gated sodium channel is expressed in nociceptors and amplifies subthreshold stimuli. It is therefore a key determinant of nociceptor excitability and selective blockers of Nav1.7 are under active development as novel analgesics (for review see [21]). Nav1.7 has been shown to be important in pathological pains states in humans [6; 64]. Homozygous loss of function mutations in Nav1.7 have been shown to cause congenital insensitivity to pain [16]. Conversely, heterozygous gain of function variants have been associated with a number of pain disorders including: inherited erythromelalgia (IEM) [67], paroxysmal extreme pain disorder (PEPD) [27] and idiopathic small fibre neuropathy [25]. In addition to these rare disorders associated with high impact variants, single nucleotide polymorphisms in Nav1.7 may have a more subtle effect in modulating the risk and severity of pain in acquired pain disorders. An example is the gain of function R1150W variant in Nav1.7 which was associated with an increased pain score in people with osteoarthritis, sciatica, phantom pain, lumbar discectomy and pancreatitis [47].

Painful DPN is one of the most common acquired neuropathic pain states in which a metabolic insult interacts with genotype; whether Nav1.7 variants are associated with neuropathic pain in DPN has not been established. We have therefore investigated whether common or rare Nav1.7 variants are associated with neuropathic pain in a cohort of patients with DPN that have undergone detailed sensory phenotyping. Detailed sensory phenotyping enables patient stratification in a manner that will reflect the pathomechanisms and that may be predictive of treatment response [4; 19]. Our aims were therefore to determine if Nav1.7 variants were related to neuropathic pain, to determine the functional effects of such Nav1.7 variants and if Nav1.7 variants could be related to somatosensory phenotype in order to improve patient stratification.

Materials and Methods

Study participants and Clinical phenotyping

Study participants were recruited as part of the Pain in Neuropathy Study (PiNS) [61]. PiNS is an observational cross-sectional multicentre study approved by the National Research Ethics Service of the UK (No.: 10/H0706/35). All study participants signed written consent before enrolment. A detailed description of the study can be found elsewhere [61] and will only briefly be described. Participants underwent a structured neurological examination, nerve conduction studies, skin biopsy for intra-epidermal nerve fibre assessment (IENFD) and a detailed quantitative sensory testing (QST) assessment. Further drug, laboratory, and clinical investigation data were retrieved from the clinical medical records. The data included nerve conduction study data and the most recent routine haematological and biochemical parameters, including HbA1c. Basic clinical parameters, such as weight, height, and blood pressures, were measured for each participant. Only study participants that had diabetes mellitus with evidence of clinical length dependant neuropathy [59] confirmed by abnormalities on either nerve conduction studies or IENFD would proceed to sequencing of their DNA (Figure S1, available online as supplemental digital content at http://links.lww.com/PAIN/A509).

A comprehensive structured upper and lower limb neurological examination was performed to detect clinical signs of a peripheral neuropathy [37; 43]. The examination included assessment of temperature, light touch and pinprick sensation, joint position proprioception, vibration perception, deep-tendon reflexes, muscle bulk, and motor power. The clinical

findings were quantified with the Toronto Clinical Scoring System (TCSS) [10] and MRC sensory sum score.

Nerve conduction tests were performed with an ADVANCE system (Neurometrix, Massachusetts, USA) and used conventional reusable electrodes. Sural sensory and peroneal motor nerve conduction studies were performed [11]. Our protocol was in line with those recommended by the American Academy of Neurology and American Association of Electrodiagnostic Medicine [22].

The determination of IENFD from skin biopsy samples is a validated and sensitive diagnostic tool for the assessment of small fibre neuropathies, including diabetic neuropathy [38]. Biopsy samples were taken in accordance with the consensus document produced by the European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the utilisation of skin biopsy samples in the diagnosis of peripheral neuropathies [38].

QST to determine somatosensory phenotypes was performed according to a previously published protocol of the German research network of neuropathic pain (DFNS) [50]. QST is a measure of sensory perception to a given stimulus. This test can show abnormalities in sensory function. QST data were entered into the data analysis system, Equista, provided by the DFNS. Equista transformed the raw QST data into z-scores thus normalising for age, sex, and the body location of testing [41; 51]. A z-score of zero is equal to the mean of the population. A score of greater or less than two standard deviations from the mean indicates gain of function or loss of function, respectively.

Orthostatic hypotension, as a marker of autonomic neuropathy, was assessed by measuring lying and standing blood pressure in accordance with established protocols [1]. Orthostatic hypotension was defined as either a 20 mm Hg reduction in systolic or a 10 mm Hg reduction in diastolic blood pressure. Survey of Autonomic Symptoms [70] is an instrument that measures the presence and impact of autonomic symptoms. It consists of 12 questions that are individually rated on a 6-point rating scale from 0 (not at all) to 5 (a lot).

Definition of neuropathic pain

Only study participants with a confirmed diabetic peripheral neuropathy proceeded to neuropathic pain (NeuP) subtyping (Figure S1, available online as supplemental digital content at http://links.lww.com/PAIN/A509). The presence of chronic NeuP caused by peripheral DPN was determined at the time of the clinical assessment and was in line with the IASP definition of neuropathic pain i.e. ‘pain caused by a lesion or disease of the somatosensory system’. The IASP/NeuPSIG grading system was used to grade the

neuropathic pain [29]. Thus participants were divided in to those with NeuP (painful DPN) and those without NeuP (painless DPN). Only study participants with chronic NeuP present for at least three months were included in the NeuP group. Study participants with non-NeuP in the extremities, such as musculoskeletal pain of the ankle, were included in the non-NeuP group.

The assessment of each study participant therefore satisfied the following criteria:

1. Pain with a distinct neuroanatomically plausible distribution i.e. pain symmetrically distributed in the extremities – completion of body map and clinical history.
2. A history suggestive of a relevant lesion or disease affecting the peripheral or central somatosensory system – diagnosis of diabetes mellitus and a history of neuropathy symptoms including decreased sensation, positive sensory symptoms e.g. burning, aching pain mainly in the toes, feet or legs.
3. Demonstration of distinct neuroanatomically plausible distribution of neuropathic pain– presence of clinical signs of peripheral neuropathy i.e. decreased distal sensation or decreased/absent ankle reflexes.
4. Demonstration of the relevant lesion or disease by at least one confirmatory test – abnormality on either the nerve conduction tests or IENFD.

Pain severity was calculated either from a pain intensity diary or the average pain over the last 24 hours. The pain intensity diary was completed over seven days, with participants recording pain at 9 am and 9 pm daily on an 11 point scale, with 0 being no pain and 10 the worst pain imaginable. The severity of NeuP from the pain diary was calculated as the mean of the pain scores obtained from the seven day pain intensity diary. Further quantification of the NeuP was calculated with the Douleur Neuropathique en 4 Questions (DN4) [7]. The DN4 is a screening tool for neuropathic pain with a score greater than four highly suggestive of neuropathic pain. Study participants completed a body map that highlighted the distribution of any pain experienced. Brief Pain Inventory (BPI) pain interference and pain severity subscales [58] were used to assess any type of pain (non-neuropathic and neuropathic) that study participants experienced and the impact of the pain on activities of daily living. BPI pain relief quantifies the relief of pain, as a percentage, that participants enjoyed after administration of an analgesic. Neuropathic Pain Symptom Inventory (NPSI) [8]), a self-administered questionnaire, evaluated neuropathic pain symptoms including evoked pain, spontaneous pain, paroxysmal pain and dysaesthesias.

Sequencing of Nav1.7

Sequencing of the coding regions of *SCN9A* was undertaken by next generation sequencing using the HaloPlex Target Enrichment System (Agilent Technologies, Santa Clara, CA, USA) and MiSeq Sequencing Platform (Illumina, Inc., San Diego, CA, USA). Sequence analysis was performed via an in-house bioinformatics pipeline utilising Burrows-Wheeler Alignment tool (BWA) [39] for mapping to the human genome and Platypus [48] for variant calling.Variants were annotated against reference sequences NM_002977.3 (mRNA) and NP_002968.1 (protein). Any variant that was both present at >1% allele frequency in the Exome Variant Database (http://evs.gs.washington.edu/EVS) and not previously reported in the literature in association with painful neuropathy was considered unlikely to be pathogenic and was not investigated further. Variants of potential interest were confirmed by Sanger sequencing by capillary electrophoresis using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA)

Plasmids and site-directed mutagenesis.

Human Nav1.7 cDNA was cloned into a modified pcDNA3 expression vector containing downstream IRES and dsRED2 sequences (*SCN9A*-IRES-DsRED) **(**Cox 2006). Human β1 and β2 subunits were cloned into pIRES2-AcGFP (SCN1B-IRES-SCN2B-IRES-eGFP) [16]. Mutations were introduced using QuikChange II XL site-directed mutagenesis kit (Agilent).

HEK293T cell culture and transfection.

Human embryonic kidney HEK-293T cells were grown in a Dulbecco's modified Eagle's culture medium (DMEM/F-12, Invitrogen) containing 10% fetal bovine serum and maintained under standard conditions at 37 °C in a humidified atmosphere containing 5% CO2. Cells were transfected using the jetPEI™ transfection reagent (Polyplus-transfection Inc.) with either WT or mutant Nav1.7 channel combined with β1 and β2 subunits (2:1 ratio). Cells were used 36 to 72 hours after transfection.

Electrophysiology.

Whole-cell patch clamp recordings were conducted at room temperature using an Axopatch 200B Amplifier, the Digidata 1550B Low Noise Data Acquisition System and the pClamp10.6 software (Molecular Devices). Data were filtered at 5kHz and digitized at 20kHz. Capacity transients were cancelled and series resistance compensated at 70-90% in all experiments. Voltage clamp experiments were performed on transfected HEK293T cells. The

extracellular solutions contained (in mM): 140 NaCl,3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.3 with NaOH (adjusted to 320 mOsm/L with glucose). Patch pipettes were filled with an internal solution containing (in mM) 140 CsF, 10 NaCl, 1 EGTA, 10 HEPES, pH 7.3 with CsOH (adjusted to 310 mOsm/L with glucose) and had a typical resistance of 2-3MΩ. Leak currents were subtracted using a P/5 protocol, applied after the test pulse. A holding potential of -100mV and an intersweep interval of 10s was used for all the protocols. Current voltage curves (I-V curves) were fitted using a combined Boltzmann and linear ohmic relationship: $I/I_{max}=G_{max}\ (V_m-E_{rev})/(1+\exp^{(V1/2-Vm)/k}$). Normalized conductance-voltage curves (activation curves) were fitted with a Boltzmann equation $G/Gmax=1/(1+\exp^{(V1/2-Vm)/K}$) were G was calculated as follows $G=I/(V_m-E_{rev})$. Similarly, the steady-state fast inactivation curves were fitted with $I/Imax=1/(1+\exp^{-(\ V1/2-Vm)/k}$) and the steady-state slow inactivation with $I/Imax=R_{in}+(1-R_{in})/(1+\exp^{-(\ V1/2-Vm)/k}$). In all the equations $V_{1/2}$ represents the half-activation and respectively half inactivation potential, V_m is the membrane potential, E_{rev} the reversal potential, k the slope factor, G the conductance and I the current at a given V_m; G_{max} and I_{max} are the maximum conductance and current respectively; R_{in} is the fraction of channels that are resistant to slow inactivation.

Structural modeling

The sequences of human NaV channels were aligned to the known structures of the cockroach Nav homologue NavPaS [54] and the Nav1.5 C-terminal domain [63] using the structural alignment tool EXPRESSO [3; 20; 44-46].

The alignment was manually edited and prepared for input into modeller (version 9.18) [42; 53; 65] by matching the sequence of the structured templates exactly, and removing the sequences of insertions. Residues 1–34, 421–722 (1–2 inter-subunit linker), 974–1168 (2–3 inter-subunit linker) were deleted and replaced by a chain break. Both available structures were used to template the model building. Calmodulin and FGF13 from the C-terminal structure were included. We generated 100 candidate models and assessed them with modellers inbuilt DOPE (M.-y. Shen and Sali 2006 [55]) score. Promising models were manually inspected and were found to show minimal variation.

Statistical Analysis.

SPSS Statistics Version 22 (IBM) and GraphPad Prism were used for statistical analysis. The QST z-scores were compared across the three groups with one-way ANOVA (LSD post hoc test). QST z-score data were expressed as mean ± 95% CI. All other data were tested for

normality with the D'Agostino-Pearson normality test and by visual inspection of their distribution. All other data were not normally distributed and reported as median with interquartile range (IQR). Data were compared between two groups with Mann-Whitney U test. Categorical data was analysed with $\chi 2$ squared test of association. Significance was set at $P = 0.05$.

Results

Study participants selection

The Pain in Neuropathy Study has recently been described in detail [61]. This study includes a cohort of 191 study participants with definite DPN i.e. diabetes mellitus with evidence of clinical length dependant neuropathy confirmed by abnormalities on either nerve conduction studies or IENFD (Figure S1, available online as supplemental digital content at http://links.lww.com/PAIN/A509). In 189 of these participants DNA was available for analysis (and these are the study participants described here). The 189 study participants with definite DPN were separated into two groups: (i) the painful DPN group comprised of 111 participants with neuropathic pain (NeuP) (ii) the painless DPN group comprised of 78 participants without NeuP. The painful DPN group all satisfied the definition of definite neuropathic as defined by the NeuPSIG/IASP grading system [29].

As previously described there were no significant differences between the two groups in terms of age, gender, BMI, blood pressure, type 2 diabetes prevalence, and time since diabetes mellitus diagnosis (Table S1, available online as supplemental digital content at http://links.lww.com/PAIN/A508). The participants with painful DPN suffered from a more severe DPN and had poorer diabetic control than the study participants with painless DPN (Table S1, available online as supplemental digital content at http://links.lww.com/PAIN/A508).

Identification of Nav1.7 variants

In both groups we then screened for rare Nav1.7 variants i.e. missense variants present at less than 1% frequency in population databases (Exome Variant Database and/or Exome Aggregation Consortium) and variants previously reported in the literature to be associated with painful neuropathy. Sequencing of the *SCN9A* gene, encoding the Nav1.7 channel, in the

111 study participants from the painful DPN group revealed the presence of 12 rare Nav1.7 variants in 10 study participants (Figure 1 and Table 1). Five of these variants were previously described in the literature as being associated with pain disorders and having a gain of function effect on Nav1.7: V991L/M932L (note that these variants are in complete linkage disequilibrium [25]); W1538R [17], R185H [31], and I739V [30]. A further variant L1267V [35] has been reported in a patient with painful neuropathy. However, it was stated that this variant did not confer hyperexcitability on DRG neurons and this patient had an additional *SCN11A* variant (and so pathogenicity is uncertain). The other six variants (I564T, K655R, S802G, K1043N, T1596I, M1852T) have never been described before in association with neuropathic pain (Table 1 and Figure 1). Most of the identified variants were only found in single study participants with the exception of R185H which was found in two study participants (Table 1). Also, there was one patient that carried four Nav1.7 rare variants V991L, M932L, W1538R and K1043T. Interestingly and in contrast to the painful DPN group, no rare Nav1.7 variants were found in the 78 study participants with painless DPN. We also screened for more common Nav1.7 variants (i.e. nonsynonymous substitution that have a frequency in population databases higher than 1%), including R1150W which was associated with an increased pain score in a previous study [47]. No statistically significant difference between the two groups could be noticed for these polymorphisms (Table S2, available online as supplemental digital content at http://links.lww.com/PAIN/A508).

Clinical description of the study participants carrying Nav1.7 rare variants

We compared the clinical characteristics of the 10 study participants carrying the Nav1.7 rare variants to the rest of the 101 study participants from the painful DPN group without the Nav1.7 rare variants.

The age, gender proportion, BMI, diabetic control (HbA1c), blood pressure, and type 2 diabetes prevalence were similar between the two groups (Table 2). The TCSS and MRC sensory sum score were also not significantly different indicating that the severity of the neuropathy does not differ. However, the study participants carrying rare Nav1.7 variants had been diagnosed for a significantly shorter duration. Also, for six of the study participants carrying rare Nav1.7 variants, the onset of neuropathic pain was at a similar time as the diagnosis of diabetes (Table 1). None of the study participants carrying a rare variant reported a family history of pain.

The study participants suffering from painful DPN completed a set of questionnaires that quantified the severity of neuropathic pain, the frequency and intensity of symptoms associated with neuropathic pain, impact on quality of life and the frequency and impact of autonomic symptoms. The study participants carrying rare Nav1.7 variants reported higher scores across all questionnaires for pain intensity and the difference reached a statistical significant value for the superficial spontaneous pain portion of the Neuropathic Pain Symptom Inventory (Table 2). Therefore, the study participants carrying rare variants reported more severe burning pain than the remaining study participants with painful DPN. There were no differences regarding the other parameters.

In order to better assess the sensory phenotype and to have more insight into pathophysiological mechanisms we also performed QST (Quantitative Sensory Testing) of the feet using the protocol developed by the DFNS [49]. Although most of the QST parameters were similar between the two groups, the z-score for pressure pain thresholds was significantly higher for the study participants with the rare Nav1.7 variants (Figure S2, available online as supplemental digital content at http://links.lww.com/PAIN/A509). In addition, the z-score for pressure pain thresholds was significantly higher for the study participants carrying rare Nav1.7 variants when compared to the study participants with painless DPN (Figure S2, http://links.lww.com/PAIN/A509). Study participants carrying rare Nav1.7 variants were therefore more sensitive to deep pressure and reported pain at lower pressures when applied to the arch of the foot.

Selection of Nav1.7 variants for functional characterization

Among all the six Nav1.7 variants not previously described in the literature, two were selected for further functional characterization: M1852T and T1596I. The selection was based on the following criteria: (i) position in important functional domains (ii) alteration of highly conserved residues (iii) important amino acid exchange (high Grantham distance) (iv) predicted as pathogenic by four different prediction algorithms (Align GVGD (http://agvgd.hci.utah.edu/), SIFT (http://sift.jcvi.org), MutationTaster (www.mutationtaster.org) and Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/)).

The M1852T substitution changes a methionine with a polar amino acid to threonine. M1852 is situated in the C-terminal part of the protein and is highly conserved in every member of the Nav family in humans (from Nav1.1 to Nav1.9) and also conserved across species (Figure

1 A and B). The variant was not present in the EVS database and was present at a very low frequency (%MAF 0.0016) in the ExAC database.

The T1596I substitution introduces an isoleucine, a hydrophobic amino acid, in place of a threonine. This amino acid sits at the top of the fourth helix of domain IV of the channel, an important helix for the voltage dependency of the channel and is highly evolutionary conserved and also conserved in all the different Nav human isoforms (Figure 1 A and C). The T1596I is present at a very low frequency in EVS and ExAC databases (%MAF 0.03 and 0.01, respectively).

Prediction programs like Align GVGD, SIFT MutationTaster, and PolyPhen-2 classified both M1852T and T1596I as "C65, likely to interfere with function", "deleterious", "disease causing" and "probably damaging", respectively.

Functional analysis of M1852T and T1596I variants

To investigate the effect that the M1852T and T1596I variants may have on the biophysical properties of the channel, we introduced these mutations into the cDNA of human Nav1.7 and expressed the mutated channels in HEK293T cells. Representative whole-cell voltage clamp currents from transfected cells are shown in Figure 2A. The mutations had no significant effect on the voltage dependence of activation of the channel (Figure 2B and D) but drastically changed the steady-state fast inactivation (Figure 2C). A 14 mV depolarizing shift of the half-inactivation potential (V1/2) could be seen for the M1852T channels and a 15mV positive shift for the T1596I channels. In addition, the mutant channels exhibited a significant increase in the slope factor for fast inactivation, the change being more prominent for the M1852T (9.1± 0.3 compared with 7.6 ± 0.3 for the T1596I and 5.6 ± 0.2 for the WT). The positive shift of the steady-state fast inactivation curve as well as the change in the slope factor led to a marked increase in the overlap between the activation and the fast inactivation (Figure 2D). This overlap predicts a larger window current for both M1852T and T1596I compared with the WT signifying that a substantially larger fraction of channels would be active at a given resting membrane potential. No significant effect on the steady-state slow activation was found (Figure 2 E).

To further characterise the effects of the M1852T and T1596I variants on the inactivation properties of Nav1.7 sodium channels we analysed the open state fast inactivation kinetics. Single exponential fits of sodium current decay demonstrated that fast inactivation occurred at the considerable slower rate for both variants (Figure 2 F). For instance, at -25 mV, wild-

type currents inactivated with a τ = 1.7 ± 0.1 ms while M1852T and T1596I currents inactivated with a significantly increased τ (3.4 ± 0.3 ms, $p<0.0001$ and 2.9 ± 0.5 ms, $p<0.05$ respectively compared with the WT). We also investigated the kinetics of the recovery from fast inactivation. Sodium channels containing the T1596I mutation recovered 3.6 times more quickly than WT channels (τ =28.7 ± 6.8 compared to τ =102.3 ± 16.7 for the WT, $p<0.005$) while no significant change was found for the M1852T channels (Figure 2 G).

One of the ways that Nav1.7 channels are thought to contribute to neuronal excitability is by generating slow depolarizations that boost subthreshold stimuli [18]. We therefore decided to examine inward currents produced by slow ramp depolarizations (Figure 2H). An increase in the ramp current could be observed for both of the mutants, the M1852T mutation having a stronger effect.

Structural insights from the M1852T and T1596I variants

To gain further insights into the effect of these two variants we constructed a structural model of the human Nav1.7 channel. The model was based on the recently published structure of the eukaryotic Nav homolog NavPS [54] and the structure of the human Nav1.5 C-terminal domain [63]. Interestingly, this model shows that the C-terminal M1852T variant is located directly beneath and in contact with the linker between domains III and IV (Figure 3 A and B). Fast inactivation of VGSCs is thought to occur via movement of this 'III-IV linker'. The position of this variant at this interface between the C-terminal domain and the α-helical part of the III-IV linker is therefore entirely consistent with its functional effects. Intriguingly, a recent structure of the Nav1.4 channel also now reveals that this interaction is state-dependent and does not occur in the open state [66]. It is therefore tempting to speculate that interactions at this interface stabilise the inactivated state and are reduced by the M1852T variant. Eitherway, this result now highlights how a dynamic interaction between these two parts of the channel is important for the control of fast inactivation.

The other variant, T1596I is located at the top of the positively-charged S4 helix within the voltage sensor of domain IV at the interface with the pore helices of domain 1 (Figures 3A and C). The voltage sensor of domain IV is also known to be an important determinant of fast inactivation: its movement being a rate limiting step for both development and recovery from fast inactivation [13]. The location of this variant is therefore also consistent with its functional effects and highlights the important role that this dynamic interface plays in the control of fast inactivation.

Discussion

The development of painful DPN is a complex multifactorial process that involves an interaction between the metabolic disturbances characteristic of diabetes mellitus, environmental factors and genotype. In this study we address the question of whether individual genetic variations in the Nav1.7 could have an impact on the development of painful DPN. We screened for Nav1.7 variants in a cohort of 189 study participants comprised of 111 with painful DPN and 78 with a painless DPN phenotype. While no rare variants could be identified in the painless group, we found a significant number (12) of rare Nav1.7 variants in ten participants from the painful DPN group. Functional characterization of two of these variants (M1852T and T1596I) demonstrated gain of function changes, consistent with an increase in neuronal excitability. This is the first study that has investigated the presence of Nav1.7 rare variants in patients with painful DPN using a cohort in which detailed phenotyping was used in order to stratify patients according to sensory profile [29]. The establishment of somatosensory phenotype allowed us to examine the differences between the ten study participants carrying Nav1.7 rare variants and the rest of the painful DPN group. Three statistical significant differences were found: (1) the study participants carrying rare Nav1.7 variants had been diagnosed for a significantly shorter duration (2) they reported more severe burning pain and (3) they were more sensitive to deep pressure and reported pain at lower pressures when applied to the arch of the foot. The absence of a clearer phenotypic distinction between the two groups is similar to a study in which erythromelalgia patients carrying the pathogenic Nav1.7 mutation I848T could not be phenotypically distinguished from those not carrying the mutation[69]. The addition of supra-threshold QST paradigms might improve our ability to differentiate mechanistically relevant subgroups [33]. Larger replication studies will be needed to determine if these phenotypic aspects could be used in the future to stratify patients for potential genetic testing. This could be relevant to future treatment choices given the major effort to develop selective small molecule blockers of Nav1.7, some of which are currently in clinical trials [68].

Among the twelve rare Nav1.7 variants identified five were already shown to be associated with painful related disorders: R185H, I739V, V991L, M932L and W1538R. The first four (R185H, I739V, V991L/M932L) were previously found in patients with idiopathic small fibre neuropathy [31]. The latter (W1538R) was identified in a patient with primary erythromelalgia [17]. One previous study also reported an association between the variants V991L/M932L (which are in complete linkage disequilibrium) and painful DPN versus

population controls [40]. In contrast to Li et al., we used painless DPN as a control group rather than normal controls without diabetes as this is the most appropriate comparison in order to address the question as to which variants promote the development of neuropathic pain in DPN. It has been hypothesised that because Nav1.7 is expressed in pancreatic β cells variants in this ion channel could confer vulnerability to injury [34].

The comparison of the published clinical phenotypes of previously reported patients with Nav1.7 variants with those from our cohort carrying the same variants revealed some differences For instance, the I739V variant was described in patients with small fibre neuropathy and severe autonomic dysfunction [30]. However, in our study we did not elicit significant autonomic dysfunction from the affected study participant. The R185H variant was also previously found in patients with small fibre neuropathy but who had minimal autonomic dysfunction [31]. In our cohort this variant was found in two patients one of which had a postural drop in blood pressure (suggesting sympathetic autonomic dysfunction) and one that didn`t. The patient from our study carrying the W1538R variant (described previously in a patient with primary erythromelalgia [17]), did not demonstrate erythromelalgia nor changes in symptomology related to changes in temperature. These findings suggest that one variant can produce different clinical pain phenotypes which will depend on environmental context. For instance in DPN the structural injury to autonomic axons as a consequence of diabetes will have a major impact on autonomic function independent of any direct effects of Nav1.7 variants on autonomic neuron excitability [31]. In our cohort none of the participants reported a family history, and only one participant reported symptoms that began before a diagnosis of diabetes was made. We therefore propose that these rare variants in Nav1.7 may act as risk factors promoting the development of neuropathic pain in the context of an environmental trigger (diabetes) rather than causing a Mendelian pain disorder.

Six of the twelve rare Nav1.7 variants identified by this study (I564T, K655R, S802G, K1043N, T1596I, M1852T) are new variants, not having been described in the literature in association with pain related disorders. However, one of them, K655R was reported in patients with febrile seizures [56]. In our case, the study participant carrying the K655R did not report a history of seizures.

An analysis of the unpublished variants using different pathogenic predictive algorithms pinpointed two of them (T1596I and M1852T) as highly likely to be pathogenic. Their electrophysiological analysis showed that these variants strongly affected the inactivation

properties of the channel. Both T1596I and M1852T exhibited a strong shift (14-15mV) of the steady-state fast inactivation curve towards more depolarised membrane potentials, increased window current, slower inactivation kinetics and, for the T1596I variant, a significant faster recovery from inactivation. All these changes are consistent with a gain of function of the channel which would most likely lead to an increase in neuronal excitability thus contributing to pain signalling.

Our structural model reveals that the position of these mutations is consistent with their functional effects. Many previous structural models of Nav1.7 have relied upon comparison to the prokaryotic Nav channels. However, we have been able to take advantage of the recent high-resolution cryoEM structure of a eukaryotic homologue, NavPS [54] to construct an almost complete model of human Nav1.7. In this model, M1852T is located in the fifth alpha helix of the C terminal domain, just below the III-IV linker, indicating possible interactions between this residue and the linker. As the III-IV linker is known to be a critical structural determinant of fast inactivation, the position of this variant not only a structural basis of our electrophysiological findings, but also highlights the dynamic role that this interface between the C terminal domain and the III-IV linker may play in fast inactivation. Likewise, the position of the T1596I variant highlights an important functional role for the interface between the voltage sensor of domain IV and the pore forming helices of domain I. Interestingly, mutation of a conserved glutamine situated at the top of S6 (Q270K) in domain I of Nav1.5 also impairs the fast inactivation [12], This glutamine is predicted to be in close proximity to T1596 and therefore suggests an important and highly-conserved role for this dynamic interface in the regulation of fast inactivation.

Compared with the other Nav1.7 variants previously associated with painful neuropathies (such as small fibre neuropathy), T1596I and M1852T appear unique. Most of the described variants in idiopathic small fibre neuropathy have relatively mild effects on fast inactivation [25]. On the other hand, Nav1.7 mutations associated with PEPD, a severe pain condition characterised by episodic pain and flare response of the sacrum, peri-ocular and mandibular regions [14; 23; 27; 36; 57; 60] have much more profound effects on inactivation (V1298F, V1299F, I1461T, G1607R, L1612P, M1627K, A1632E). Two of these mutations G1607R, L1612P are also located in S4 of domain IV with the others in the S4-S5 linker of domain III (V1298F, V1299F), S4-S5 linker of domain IV (M1627K, A1632E) or part of the IFM motif in linker III-IV (I1461T). However, the M1852T variant we describe here is the first mutation in the C-terminal domain of Nav1.7 reported to have a major effect on inactivation and may

also provide evidence for a possible state-dependence of the interaction of the III-IV linker with the C-terminal domain during inactivation.

Although broad generalisations can be drawn when comparing the biophysical impact of Nav1.7 variants with clinical phenotype (for instance that IEM is associated with enhanced channel activation and PEPD with impaired channel inactivation) we still have an incomplete understanding of how channel dysfunction causes specific pain phenotypes. Certain Nav1.7 variants have also been shown to cause the degeneration of DRG axons *in vitro* particularly under conditions of metabolic stress [24; 52] and so an interesting topic for future studies will be whether Nav1.7 variants may not only impact on pain phenotype in DPN but also neuropathy progression.

In conclusion, this study reveals an important link between painful DPN and Nav1.7 suggesting that rare Nav1.7 variants may predispose patients with diabetic neuropathy to developing neuropathic pain. Despite the challenges prospective studies of diabetes and its complications would be very helpful in extending these findings. Better understanding of genetic variability in neuropathic pain disorders combined with improved sensory phenotyping should also improve patient stratification for future clinical trials and help target therapy more appropriately.

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Conflict of interest

Conflict of interest

ASCR undertakes consultancy and advisory board work for Imperial College Consultants- in the last 36 months this has include remunerated work for: Spinifex, Abide, Astellas, Neusentis, Merck, Mitsubishi, Aquilas, Asahi Kasei, Galapagos, Toray, Quartet, Relmada, Novartis and Orion. ASCR was the owner of share options in Spinifex Pharmaceuticals from which personal benefit accrued upon the acquisition of Spinifex by Novartis in July 2015 and from which future milestone payments may occur.

ASCR is named as an inventor on patents: Rice A.S.C., Vandevoorde S. and Lambert D.M Methods using N-(2-propenyl)hexadecanamide and related amides to relieve pain. WO 2005/079771; Okuse K. et al Methods of treating pain by inhibition of vgf activity EP13702262.0/ WO2013 110945. DLB has undertaken consultancy and advisory board work for Oxford innovation- in the last 36 months this has included renumerated work for: Abide, Biogen, GSK, Lilly, Mitsubishi Tanabe, Mundipharma, TEVA and Pfizer.

All other authors declare no conflict of interest.

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Figure legends

Figure 1 – Nav1.7 variants identified in study participants with painful diabetic peripheral neuropathy

A. Schematic of Nav1.7 channel topology. The variants previously reported in the literature as associated with painful neuropathy are represented with blue dots and the newly identified variants with red ones.
B. Sequence alignment of human Nav1.1-Nav1.9 channels and of Nav1.7 channel in different species showing the conserved M1852 amino acid in red.
C. Sequence alignment of human Nav1.1-Nav1.9 channels and of Nav1.7 channel in different species showing the conserved T1596 amino acid in red.

Figure 2 – Effect of M1852T and T1596I variants on the biophysical properties of Nav1.7 channels

A. Representative currents elicited from a holding potential of -100 mV to different test pulse potentials (50ms) ranging from -80 to 40mV in 5mV increments, for the WT (black), M1852T (red) or T1596 (blue) channels.

B. Normalized peak current-voltage relationship curves from traces in panel A for the WT (black dots, $V_{1/2}$ = -29.9 ± 1.3, k = 5.7 ± 0.3, n=19), M1852T (red squares, $V_{1/2}$ = -27.6 ± 1.3, k = 6.3 ± 0.5, n=11) or T1596 (blue triangles, $V_{1/2}$ = -26.6 ± 2.4, k = 5.5 ± 0.6, n=9) channels.

C. Steady-state fast inactivation curves for the WT (black dots, $V_{1/2}$ = -86.6 ± 1.4, k = 5.6 ± 0.2, n=18), M1852T (red squares, $V_{1/2}$ = 72.5 ± 3, k = 9.1± 0.3, n=11, $P \leq 0.0001$) or T1596 (blue triangles, $V_{1/2}$ = 71.2 ± 2.2, k = 7.6 ± 0.3, n=9, $P \leq 0.0001$) channels. Currents were elicited with test pulses to -10 mV following 500 ms inactivating prepulses.

D. Overlapping voltage dependence of steady-state activation and steady-state fast inactivation. The inset shows an enlargement of the overlapping area representing the window current for the WT(black), M1852T(red) and T1596I(blue) channels.

E. Steady-state slow inactivation curves for the WT (black dots, $V_{1/2}$ = -61.1 ± 2.8, k = 13.7 ± 0.5, n=12), M1852T (red squares, $V_{1/2}$ = -67.5 ± 3.8, k = 12.9 ± 0.8, n=8) or T1596 (blue

triangles, $V_{1/2}$ = -60.9 ± 2.4, k = 11.7± 0.7, n=9) channels. Currents were elicited with test pulses to -10 mV following 30s inactivating prepulses and a pulse to -120mV to remove fast inactivation.

F. Open-state fast-inactivation kinetics for the WT (black dots, n=19), M1852T (red squares, n=11) or T1596 (blue triangles, n=9) channels, measured by fitting the current decay of the traces in A with a single exponential function.

G. Recovery from inactivation for the WT (black dots, τ =102.3 ± 16.7, n=14), M1852T (red squares, τ =106.3 ± 21.8, n=9) or T1596 (blue triangles, τ =28.7 ± 6.8, n=8) channels measured using two -10 mV test pulses lasting 20 ms applied from a holding potential of -100 mV and separated by increasing durations

H. Mean ramp currents for the WT (black, n=11), M1852T (red, n=7) or T1596 (blue, n=8) channels. The currents were evoked by depolarizing the membrane potential at a rate of 0.2mV/ms from -100 to 0 mV. The response has been rescaled as the percentage of the maximal peak inward current obtained from traces in panel A.

Data information: In (B–G), data are presented as mean ± SEM. Statistical analysis were performed using one-way ANOVA combined with Dunnett post hoc analysis for multiple comparisons. p values given compared with the WT. $V_{1/2}$ represents the half-activation and respectively half inactivation potential, k the slope factor,

Figure 3 – Nav1.7 channel structural model

A. Side view of the Nav1.7 structural model showing the M1852 (magenta) and T1596 (yellow).

B. Enlargement of the region containing the M1852 residue; note the proximity to the III-IV linker

C. Enlargement of the region containing T1596; note the proximity to the S6 of domain I

The transmembrane domains D I, D II, D III, D IV are represented in dark blue, light blue, cyan and aquamarine respectively. The cytoplasmic linker III-IV (L III-IV) is in dark red and the C-terminal domain (CTD) in cream.

Table legends

Table 1. Clinical description of the study participants with painful diabetic neuropathy carrying Nav1.7 rare variants

The 24 hour pain score was the average pain intensity over the preceding 24 hours rated on a 11-point Likert scale with 0 rated as “no pain” and 10 rated as “worst pain imaginable”. The DN4 score is a screening toll for neuropathic pain with a score greater than four suggestive of

neuropathic pain. Postural blood pressure drop was used as a measure of autonomic dysfunction. Almost all participants were prescribed analgesics that included at least one drug recommended for the treatment of neuropathic pain. The BPI pain severity quantified the severity of pain and the BPI pain relief was quantified the analgesic effect enjoyed by the study participants.

MAF = minor allele frequency, ExAC= Exome Aggregation Consortium

Table 2. Summary of variables that were compared between the study participants with Nav1.7 rare variants and the rest of the painful diabetic peripheral neuropathy group

Data shown as median (interquartile range) and analysed by Mann-Whitney U Test. Categorical data were analysed by Fischer's exact test, and values and percentages are shown.

Symbols reflect the differences between the respective groups: * $P < 0.05$

Study participant	Nav1.7 variant	MAF (ExAC)	PMID	Age	Gender	Ethnicity	Diabetes type	Age at diabetes diagnosis	Age at pain onset	Toronto Clinical Score	24h pain	DN4	Analgesia	BPI Pain Relief	Postural blood pressure drop
1	R185H	0.33	Han et al. 2012 22826602	48.4	m	Asian	2	31.7	44.7	7	4	4	Pregabalin	Not completed	No
2	R185H	0.33	Han et al. 2012 22826602	64.9	m	White European	2	62.0	62.0	15	7	5	Pregabalin, Duloxetine, Capsaicin ointment, Co-codamol	40	yes
3	I564T	0.0025	-	62.5	m	White European	2	51.2	52.2	13	9	7	Pregabalin, paracetamol	70	yes
4	K655R	0.19	-	53.5	f	White European	2	40.2	51.5	12	9	8	Carbamazapin, Codeine, Ibuprofen, paracetamol	100	no
5	I739V	0.39	Han et al. 2012 22539570	75.7	m	White European	2	64.0	72.2	18	3	5	Gabapentin, Co-codamol	100	No
6	S802G	0.29	-	41.4	m	Other	1	38.7	38.6	9	7	6	Gabapentin, tramadol	Not completed	No
7	M932L	2.95	Faber 2011	49.8	m	White Europea	2	37.2	49.3	14	8	6	Oxycodone, naproxen, lorazepam,	50	No

			21698661										
	V991L	3.11	Faber 2011 21698661		n						duloxetine		
	K1043N	0	-										
	W1538R	0.21	Cregg et al 2013 23292638										
8	L1267V	0.14	Hunag et al 2014 24776970	72.1f	White European	2	68.8	64.9	12	6	9	None	Non applicable	no
9	T1596I	0.01	-	69.0m	White European	2	61.3	61.3	12	7	6	Amitriptyline, Tramadol	30	No
10	M1852T	0.0016	-	59.5f	White European	2	55.6	56.6	11	7	7	Pregabalin, Duloxetine	20	No

Table 1. Clinical description of the study participants with painful diabetic neuropathy carrying Nav1.7 rare variants

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	Painful diabetic neuropathy	
	With rare Nav1.7 variants	Without rare Nav1.7 variant
Number	10	101
Male	7 (70 %)	70 (69.3 %)
Age (years)	61 (49.5-69.8)	67.6 (57.7-73.)
HbA1c (%)	8.4 (7.6-9.5)	7.5 (6.8-8.9)
Type 2 diabetes	9 (90%)	90 (89.1%)
Duration of diabetes (years)	**9.5 (3.2-12.8)**	**14.7 (8.5-22.7) ***
BMI (kg/m^2)	30.7 (28.5-36.7)	30.5 (26.4-36.1)
Standing BP	132/86 (115/64-149-88)	141/78 (128/67-153/83)
Lying BP	145/80 (120/66-156-94)	146/78 (135/71-156/85)
Orthostatic hypotension	2 (20%)	25 (26.9%)
MRC sensory sum score	10.5 (5-12.8)	6 (3-12)
TCSS Total (adjusted)	11 (9.5-13.3)	11 (8-13.5)
TCSS Symptoms score (adjusted)	3 (2-4)	3(2-4)
TCSS Sensory examination	4 (3-5)	3 (2-5)
TCSS Examination	8.5 (7-9.3)	8 (5-10.5)
IENFD (fibres/mm)	0.6 (0-1.4)	0.9 (0.3-1.6)
Pain over last 24 hours	7 (5.5-8.3)	5.5 (4-8)
Seven day pain diary	6.5 (4.9-7.2)	5 (2.9-6.8)
DN4	6 (5-7.3)	6 (4-7)

BPI Pain Interference	5.8 (7.9-2.8)	4.1 (2.5-6.1)
BPI Pain Severity	5.4 (3.8-7.1)	4.5 (2.5-6.3)
BPI Pain relief (%)	50 (30-100)	40 (0-60)
NPSI Total	5.2 (2.7-5.7)	3 (1.6-4.6)
NPSI Deep Spontaneous Pain	3 (0-4.5)	1 (0-4)
NPSI Superficial Spontaneous Pain	**7 (5-8.5)**	**4 (0-7) ***
NPSI Evoked Spontaneous Pain	3 (1.2-4.9)	1.7 (0-4.3)
NPSI Paraesthesiae	8 (3.5-8)	4.3 (1.9-8)
NPSI Paroxysmal Pain	4.5 (3.3-6.3)	3.5 (0.4-6.1)
Autonomic symptom score	5 (1.5-7)	4 (3-6)
Autonomic impact score	11 (6.5-17)	11 (6-17)

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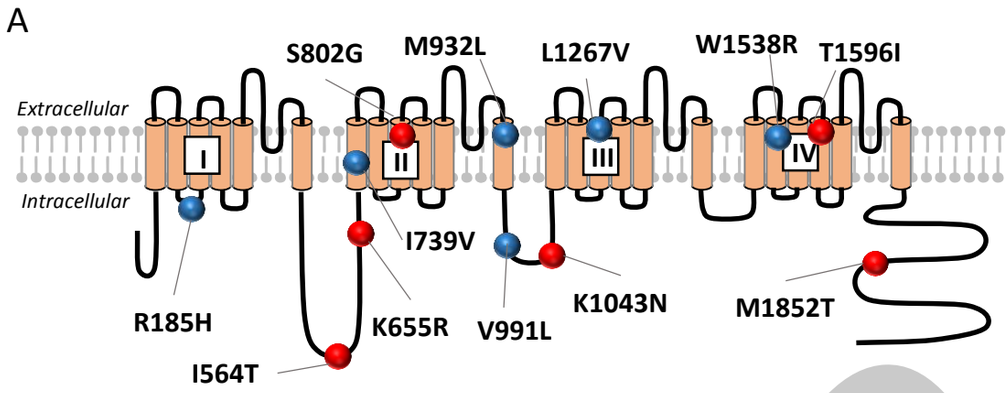
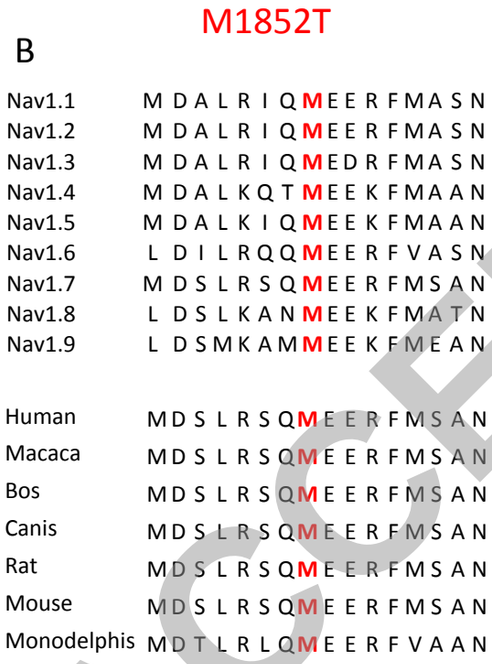
Symbols reflect the differences between the respective groups: * $P < 0.05$.





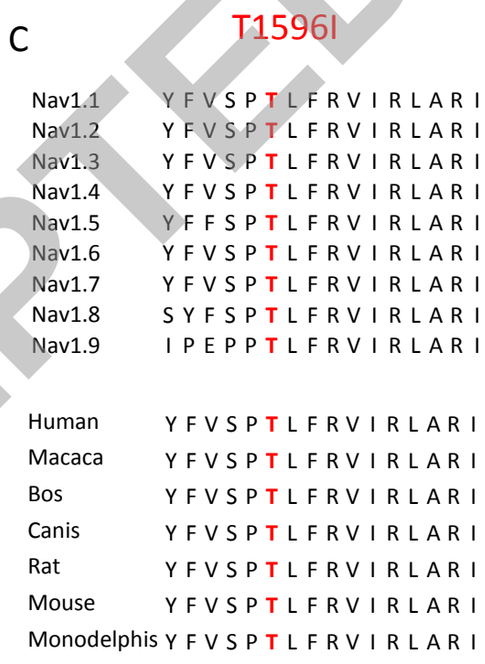


Figure 1